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Genetic structure in *Hypericum perforatum* L. population

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Abstract

Background and Purpose: *Population structure in* Hypericum perforatum *L., a hybrid apomictic plant species of medicinal importance, was studied using RAPD markers. The total of 109 samples collected from various locations in Croatia were compared on the basis of RAPD profiles obtained by amplifications with six primers.*

Materials and Methods: *Total cellular DNA was extracted from the samples collected from 8 locations in Croatia. PCR amplification was performed using 6 decamer oligonucleotide primers. Allele frequencies were computed according to Bayesian method with non-uniform prior distribution. The number of polymorphic loci (95% criterion) and Nei's gene diversity for population and the group of subspecies were calculated using program package AFLP-SURV 1.0.*

Results and Conclusions: *The results obtained show gene diversity in* H. perforatum *L. in a range between 0.121 and 0.301. Three populations had significantly lower level of diversity, four populations had slightly higher, while one population was polymorphic. Higher polymorphism of one population as well as variations in values between different populations may be explained by residual fertility and outcrossing which may have occurred in some populations. High fixation index (0.331) indicates limited gene flow as the consequence of prevaling apomixis.*

INTRODUCTION

The determination of the plant population genetic structure is of great importance in the development of strategies for collecting and conserving plant materials as genetic resources, as well as in improvement of their utilization. Medicinal plants are sources of numerous secondary metabolites, and germplasm conservation in their natural habitats is of particular importance.

Hypericum perforatum L. (*Guttiferae*) is a medicinal plant abounding with secondary metabolites which have clinically proven anti-inflammatory, antiviral, antimicrobial, antifungal, citotoxical effects and also antidepressant activity *(2, 4, 16, 17, 29)*. *H. perforatum* L. is a glabrous erect perennial herb recognizable by simple, opposing, ovate to linear leaves sprinkled with oil translucent glands. Bright yellow flowers consist of five assymetric petals with hypericin containing black nodules *(23)*. *H. perforatum* L. is the hybrid apomictic species *(15, 24, 25)* widespread in Europe (except in extreme north), Asia, Northern

Africa, as well as in North America, New Zealand and Australia where it was introduced as a very resistant weed *(5)*.

To our knowledge, there is no report on the application of molecular markers to study the population structure of *H. perforatum*. In this study, populations of *H. perforatum* in Croatia were examined using random amplified polymorphic DNA (RAPD, *31, 32*). RAPD fragments usually provide visible resolution between closely related individuals and therefore particularly applicable for the study of within-population variation *(11)*. The use of these markers in investigation of reproduction in *H. perforatum (1)* and somaclonal and natural DNA variation in *H. perforatum (7)* confirmed the possibility for identification and differentiation of individuals by this method. In order to avoid incompleteness of genotypic information due to dominant character of the RAPD markers, Lynch and Milligan's *(14)* estimators were used. In the presented study, we assumed homology of all comigrating bands, since individuals of the same species were compared. In support, many Southern analyses which are often used to test homology, confirm that apparently identical bands are homologous in samples of closely related species *(11, 22, 26, 33, 34)*. However, it is important to mention that non-homologous similar-sized fragments are in minority when the number of polymorphic bands is high (as in our investigation), thus their contribution to error in the data was taken as minor. Repeated PCR reactions for each sample/primer combination in the strictly constant conditions with positive and negative control were performed to achieve and test reproducibility and repeatability of RAPD amplification profiles *(8, 32)*.

MATERIALS AND METHODS

Plant material

109 individuals of *H. perforatum* were sampled from 8 locations in Croatia covering Adriatic coast, Dinaric alps and Pannonic regions (Figure 1). Each population includes 12 – 16 individuals which were selected randomly over at least 3 – 4 ha area with homogenous environment. The distance between the collected individuals was at least 20 m to avoid possible effect of clone caused by vegetative and seed apomixis.

DNA extraction and amplification

Total cellular DNA was extracted from 100 – 140 mg fresh or frozen leaves following the procedure of Doyle and Doyle *(6)* modified as reported in Petit *et al (21)*.

Of a total of 20 random decamer oligonucleotide primers which were preliminarily screened for the amplification of RAPD fragments, six were selected on the basis of the level of diversity, repeatability and clearness of the obtained products. The chosen primers were: OPJ 01, OPJ 08, OPB 03, OPB 08, OPB 16 and OPB 20 (Operon Technologies, Alameda, CA, USA). For PCR amplification, 8 to 12 nanograms of total DNA per sample were

Figure 1. Hypericum perforatum *L. collected samples from Croatia. 1 – Kutjevo (16 samples), 2 – Kalnik (13 samples), 3 – Plitvice (11 samples), 4 – Baške Oštarije (14 samples), 5 – Funtana (14 samples), 6 – Učka (14 samples), 7 – Knin (12 samples), 8 – Solin (15 samples).*

used. Amplification reactions were performed in the volume of 25 µl containing 2.5 mM MgCl, 60 µM of each nucleotide, 5 pmol of primer and 0.7 units of Taq DNA polymerase (Gibco BRL). Amplifications were carried out in a Perkin Elmer 2 400 thermal cycler programmed as follows: the first denaturation (4 min at 94 °C), then 40 cycles of denaturation (60 sec at 94 °C), annealing (60 sec at 36 °C), elongation (120 sec at 72 °C) and then final elongation step of 10 min at 72 °C. Each reaction was repeated twice on the same samples.

Electrophoresis

The obtained amplification products were separated on 1.4 % agarose gel in TAE buffer that contained 0.5 µg/ml ethidium bromide for 4.5 hours at 70 V. A 500 bp DNA Ladder (Gibco BRL) was used for band determination. After electrophoresis, the gel was viewed under UV light and the bands were scored using Image Master system (VDS Pharmacia Tech).

Data analysis

Each PCR product represents one single biallelic locus, named by the primer used and the molecular mass in base pairs (bp). Products (bands) were scored as presence (1) or absence (0) and data matrix of different RAPD phenotypes was assembled.

Statistical analyses of RAPD products were based on the assumption that comigrating fragments represent homologous loci. Because of RAPD data dominant nature, Lynch and Milligan's *(14)* corrected allele frequencies were used for calculation of gene diversity parameters. Allele frequencies were computed according to Bayesian

TABLE 1

Used primers and obtained RAPD fragments for the 109 individuals studied.

Primer	Sequence 5'→3'	Number of fragments	Size (bp) min – max
OPB 03	CATCCCCCTG	18	400–2500
OPB 08	GTCCACACGG	15	600–2900
OPB 16	TTTGCCCGGA	9	700–2300
OPB 20	GGACCCTTAC	17	700–3000
OPJ 01	CCCGGCATAA	9	800–2050
OPJ 08	CATACCGTGG	17	600–2900

TABLE 2

Genetic diversity within 8 Croatian populations of *H. perforatum* L. based on RAPD markers.

Population	N	l	pl	pl (%)	He (SE)
Kutjevo	16	81	40	49.4	0.184 (0.021)
Kalnik	13	82	70	85.4	0.301 (0.019)
Plitvice-Korenica	11	82	59	72.0	0.269 (0.021)
Baške Oštarije	14	82	57	69.5	0,263 (0.020)
Funtana	14	82	59	72.0	0,262 (0.019)
Učka	14	82	50	61.0	0.246 (0.028)
Knin	12	82	50	61.0	0.188 (0.019)
Solin	15	82	39	47.6	0.121 (0.017)

N – number of samples, l – number of loci scored, pl – number of polymorphic loci (5% level), pl% – percentage of polymorphic loci, He – gene diversity (Nei 1973), SE – standard error of He.

method with non–uniform prior distribution *(32)*. The number of polymorphic loci (95% criterion) and Nei's gene diversity *(17)* for population and group of subspecies were calculated using program package AFLP-SURV 1.0 *(30)*.

RESULTS AND DISCUSSION

All the primers used produced multiple band profiles consisting of several common bands and a varying number of uncommon or sporadic bands. In repeated reactions the primers give reproducible band profiles. A total of 82 amplification products were scored, 9–18 products per primer. The products ranged from 400 bp to 3000 bp. The number of bands scored from electrophoretical pattern for the each primer used are presented in TABLE 1.

Gene diversity in *H. perforatum* L. populations is in a range between 0.121 and 0.301 (TABLE 2). Three populations had significantly lower level of diversity: Adriatic populations from Solin and Knin (0.121 and 0.188) as well as Pannonic from Kutjevo (0.188) (TABLE 2 and 3). Four populations, two from Istra (Učka 0.246 and Funtana 0.262) and two from middle Croatia (Plitvice 0.269 and Baške oštarije 0.263), have similar diversity, while population from Kalnik had higher level of diversity (0.301). The total gene diversity is 0.342, within population component is 0.229 (77 %) while between populations diversity is 0,113 (33 %). NEI *(18)* suggested the use of gene diversity parameter as a measure of the level of genetic diversity for any organism regardless of its mode of reproduction. Outcrossing, windpollinating, woody and longliving species as well as outcrossing, perennial mainly herbaceous and insectpollinating species have high values of Nei's gene diversity, for example: *Populus grandidentata*: 0.350 *(13)* and *Brassica oleracea*: 0.490 *(12)*. Gene diversity of annual or short living perennial, mainly selfing species is lower, for example: *Hordeum spontaneum*: 0.116 – 0.203 *(20)*. On the other hand, for this type of plants there is evidence that some of the sampled populations may have a very high level of polymorphism as it is a case for *Medicago trancatula (3)* and Arctic triploid grass *Puccinellia x phryganodes (10)*. In our investigation

TABLE 3

T – test based on gene diversities of 8 Croatian population of *H. perforatum* L. ns = non significant; * 0.01 < p < 0.05; ** 0.001 < p < 0.01; *** p < 0.001.

	Kutjevo	Kalnik	Plitvice-Korenica	Baške Oštarije	Funtana	Učka	Knin	Solin
Kutjevo	–							
Kalnik	**	–						
Plitvice-Korenica	**	n.s.	–					
Baške Oštarije	**	n.s.	n.s.					
Funtana	**	n.s.	n.s.	n.s.	–			
Učka	*	n.s.	n.s.	n.s.	n.s.	–		
Knin	n.s.	**	**	*	*	*	–	
Solin	*	***	**	***	***	**	*	–

TABLE 4

Total gene diversity of *H. perforatum* L. samples from Croatian area based on RAPD markers.

Ht (Hs+Hb)	Hs (SE) %	Var Hs	Hb (SE) %	Var Hb	F_{ST} (SE)	Var F_{ST}
0.342	0.229 (0.021) 77	0.00044	0.113 (0.022) 33	0.00046	0.331 (0.159)	0.02537

Ht – total gene diversity, Hs – average gene diversity within subspecies, Var Hs – variance of Hs, Hb – average gene diversity between subspecies, Var Hb – variance of Hb. F_{ST} – Wright's fixation index, Var F_{ST} –variance.

variation of the gene diversity values between populations was also noticed. Three populations had significantly lower level of diversity, four populations had slightly higher similar diversity, while one population was polymorphic. These results suggest that some populations of the genofond of the same selfing species may be more diverse than others as the consequence of different percent of outcrossing that occurs or different population ages as Shoen and Brown *(27)* established. In addition, some populations, due to environmental factors, may contain sufficient genetic variation for adaptive responses, which may be present in *H. perforatum* since this species is a weed with high colonizing ability.

Wright's fixation index (Fst) obtained from the data for Croatian population is 0.331 (Table 4). Heywood *(9)* emphasized that Fst is a good measure of spatial isolation and differs according to gene flow. He also quoted some Fst values obtained in investigations and concluded that obligate outcrossers have Fst ranging from 0.004 to 0.08, while for selfers the values are between 0.026 to 0.78. According to the above mentioned interpretation, high Fst of *Hypericum perforatum* L. populations indicates limited gene flow as a consequence of prevailing apomixis. Short distance of seed dispersal also has potentional influence on gene flow.

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